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# Blood and liver telomere length, mitochondrial DNA copy number, and hepatic gene expression of mitochondrial dynamics in mid-lactation cows supplemented with L-carnitine under systemic inflammation

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## ABSTRACT

The current study was conducted to examine the effect of L-carnitine (LC) supplementation on telomere length and mitochondrial DNA copy number (mtDNAcn) per cell in mid-lactation cows challenged by lipopolysaccharide (LPS) in blood and liver. The mRNA abundance of 31 genes related to inflammation, oxidative stress, and the corresponding stress response mechanisms, the mitochondrial quality control and the protein import system, as well as the phosphatidylinositol 3-kinase/protein kinase B pathway, were assessed using microfluidics integrated fluidic circuit chips (96.96 dynamic arrays). In addition to comparing the responses in cows with or without LC, our objectives were to characterize the oxidative and inflammatory status by assessing the circulating concentration of lactoferrin (Lf), haptoglobin (Hp), fibrinogen, derivates of reactive oxygen metabolites (dROM), and arylesterase activity (AEA), and to extend the measurement of Lf and Hp to milk. Pluriparous Holstein cows were assigned to either a control group (CON, n = 26) or an LC-supplemented group (CAR; 25 g LC/cow per day; d 42 ante partum to d 126 postpartum (PP), n = 27). On d 111 PP, each cow was injected intravenously with LPS (*Escherichia coli* O111:B4, 0.5 μg/kg). The mRNA abundance was examined in liver biopsies of d −11 and +1 relative to LPS administration. Plasma and milk samples were frequently collected before and after the challenge. After LPS administration, circulating plasma fibrinogen and serum dROM concentrations increased, whereas AEA decreased. Moreover, serum P4 initially increased by 3 h after LPS administration and declined thereafter irrespective of grouping. The Lf concentrations increased in both groups after LPS administration, with the CAR group showing greater concentrations in serum and milk than the CON group. After LPS administration, telomere length in blood increased, whereas mtDNAcn per cell decreased; however, both remained unaffected in liver. For mitochondrial protein import genes, the hepatic mRNA abundance of the translocase of the mitochondrial inner membrane (*TIM*)-*17B* was increased in CAR cows. Moreover, *TIM23* increased in both groups after LPS administration. Regarding the mRNA abundance of genes related to stress response mechanisms, 7 out of 14 genes showed group × time interactions, indicating a (local) protective effect due to the dietary LC supplementation against oxidative stress in mid-lactating dairy cows. For mtDNAcn and telomere length, the effects of the LPS-induced inflammation were more pronounced than the dietary supplementation of LC. Dietary LC supplementation affected the response to LPS primarily by altering mitochondrial dynamics. Regarding mRNA abundance of genes related to the mitochondrial protein import system, the inner mitochondrial membrane translocase (TIM complex) seemed to be more sensitive to dietary LC than the outer mitochondrial membrane translocase (TOM complex).

**Key words:** inflammation, mitochondrial dynamics, L-carnitine, telomere length

## INTRODUCTION

Intravenous injection of bacterial LPS triggers an inflammatory response by activating the immune system (e.g., review by Bradford et al., 2015). As part of this process, immune cells likely switch their metabolic strategies between fatty acids (**FA**) oxidation and gly-

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colysis during activation, as known for T cells, or, in opposite direction, for macrophages (Hosomi and Kunisawa, 2020). L-Carnitine (**LC**) is essential for the oxidation of FA with chain lengths of 14 or more carbons by forming long-chain acylcarnitineesters, which can be imported into the mitochondria (carnitine shuttle; Nelson et al., 2017). Dietary supplementation of dairy cows with LC may support the oxidation of dietary fat and FA mobilized from adipose tissue during early lactation, which may reduce the likelihood of developing metabolic disorders such as fatty liver syndrome and ketosis (Carlson et al., 2007). In addition, LC supplementation was demonstrated to lower markers of inflammation and indicators of oxidative stress in humans (Fathizadeh et al., 2020).

Administration of LPS has been demonstrated to increase lipolysis in humans (Rittig et al., 2016), and increased circulating concentrations of FA were demonstrated in mid-lactation cows whereby the response was smaller in cows receiving LC than in the control cows without supplementation (Meyer et al., 2021). The effects of LPS on liver triglyceride content seem to depend on the stage of lactation: in early lactation, LPS was shown to increase hepatic lipid accumulation in dairy cows (Graugnard et al., 2013), whereas a decrease was reported for mid-lactation cows (Alaedin et al., 2021).

Mitochondria, the main source of energy production in mammalian cells, produce reactive oxygen species (**ROS**); however, excessive ROS production can impair mitochondrial function (Cadenas and Davies, 2000). Several processes such as cytokine secretion, proliferation, differentiation, increased β-oxidation, and immune responses can upregulate ROS production (Hensley et al., 2000; Tahara et al., 2009). In humans, mitochondrial dysfunction has been linked to oxidative stress and telomere shortening (Gonzales-Ebsen et al., 2017). In general, high ROS levels resulting from dysfunctional mitochondria can accelerate telomere shortening, one of the major factors in the aging process (Barja, 2014). Telomeres are short, repetitive nucleotide sequences at the ends of chromosomes that protect chromosome integrity (Blackburn, 1991). Telomeres shorten with each cell division and when they reach a critical size, they initiate cell senescence, which can lead to cell death; therefore, telomeres are considered as biological markers reflecting cellular aging processes (von Zglinicki and Martin-Ruiz, 2005).

During metabolic or environmental stress, mitochondria change their size by fusion and fission to maintain mitochondrial function (Lee et al., 2004). In addition, energetically demanding processes, such as inflammation, lead to the adaptation of mitochondrial functions in different cell types (Hock and Kralli, 2009). Mitochondria have their own genome and the copy number of mitochondrial DNA (**mtDNA**) reflects the abundance of mitochondria in a cell (Al-Kafaji and Golbahar, 2013). However, mtDNA encodes only about 1% of all mitochondrial proteins; the remaining proteins are synthesized as precursor proteins in the cytosol and must be imported into mitochondria (Harbauer et al., 2014). These precursor proteins are imported into the outer and inner mitochondrial membrane by a specific protein import system (i.e., the outer and inner mitochondrial membrane translocation proteins; TOM/TIM complex; Harbauer et al., 2014). In humans, mitochondrial quality control is considered an adaptive mechanism to ensure mitochondrial homeostasis (Wang and Zhou, 2020) and mitochondrial dysfunction may contribute to health disorders such as insulin resistance (Jheng et al., 2012). Mitochondrial quality control involves several processes, such as mitochondrial biogenesis, dynamics, mitophagy, and mitochondrial cell death (Picca et al., 2018).

With this background, we hypothesized that dietary supplementation with LC will affect the response to an inflammatory stimulus in terms of hepatic mitochondrial function, telomere lengths, the mtDNA copy number (**mtDNAcn**) per cell in blood leukocytes and liver, and hepatic expression of genes related to mitochondrial quality control and protein import system and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway in mid-lactation dairy cows. In addition to comparing the respective responses in cows with or without LC, our objective was to characterize oxidative and inflammatory status by determining circulating concentrations of lactoferrin (**Lf**), haptoglobin (**Hp**), fibrinogen, derivatives of reactive oxygen metabolites (**dROM**), and arylesterase activity (**AEA**) and extending the measurement of Lf and Hp to milk. In addition, it was hypothesized that inflammatory status would affect ovarian activity in mid-lactation cows because progesterone (P4) secretion from the bovine corpus luteum of cows was decreased after LPS administration (Lüttgenau et al., 2016). Therefore, in the present study, we aimed to analyze serum P4 as a proxy for luteal activity. The results could fill the knowledge gap on the effects of inflammatory processes on mitochondria and their regulatory pathways in lactating dairy cows.

## MATERIALS AND METHODS

The animal experiment was performed at the experimental station of the Institute of Animal Nutrition of the Friedrich-Loeffler Institut (Braunschweig, Ger-

many). The experiment was conducted following European regulations for the protection of experimental animals and was approved by LAVES (Lower Saxony State Office for Consumer Protection and Food Safety, Germany, AZ33.19–42502–04–16/2378).

### Animals and Experimental Design

The animal experiment was previously reported by Meyer et al. (2021) describing the lactation performance, energy and mineral status, the circulating concentrations of carnitine and its metabolites, blood gas values, clinical findings as well as leukocyte counts, cortisol, and Hp as indicators for the acute-phase reaction (**APR**, Supplemental Figures S1 and S2, https://doi.org/10.6084/m9.figshare.14822418.v3, Ghaffari, 2023). Briefly, 53 pluriparous German Holstein dairy cows were assigned to either a control group (**CON**, n = 26) or an LC group (**CAR**, n = 27; Figure 1). Power test analysis was performed with a significance level of 0.05 and a desired statistical power of 0.80. Based on previously published values and accounting for telomere length, a sample size of 5 cows per treatment was predicted (Laubenthal et al., 2016b). While no specific power test for targeted gene expression was performed, previous studies (Dann et al., 2005; Loor et al., 2007; Sun et al., 2019) suggest that a sample size of 25 cows per treatment group would be sufficient to detect statistical differences in gene expression and molecular analyzes. Animal groups were balanced by lactation number (2–5), BCS (2.5–4.75), BW (568–1,008 kg), and FCM yield from the previous lactation. Both groups received increasing portions of concentrate (30%–50%) in the first 2 wk of lactation and 50% concentrate until the end of the experiment based on DM according to the recommendations of the German Society for Nutrition Physiology (GfE, 2001). Depending on the group, cows received either 125 g of a rumen-protected LC product corresponding to 25 g LC per cow per day (Carneon 20 Rumin-Pro, Kaesler Nutrition GmbH, Cuxhaven, Germany) or a rumen-protected fat product (BergaFat F-100 HP, Berg + Schmidt GmbH and Co KG) in equal amounts from d 42 ante partum (**AP**) until the end of the experiment on d 126 postpartum (**PP**). On d 111 PP, cows were injected intravenously with 0.5 μg LPS (*Escherichia coli* O111:B4, Sigma-Aldrich) per kg of BW. The cows were not inseminated during the lactation period studied and were thus not pregnant when the LPS challenge was performed.

### Blood and Tissue Sampling

Blood was sampled from a Vena jugularis externa frequently before and after the LPS challenge; the collection and processing of the blood samples were described in detail by Meyer et al. (2021). The present part of the experiment, which included the immune challenge, covered the period from d 110 to 126 PP. Blood samples were collected at the following time points: d 100 and d 110 PP (−11 and −1 d relative to LPS administration), d 111 PP, 0.5, 1, 2, 3, 4, 6, 9, 12, 24, 48, and 72 h post-LPS injection (**p.i.**), and d 118 and 126 PP, corresponding to 7 and 15 d p.i., by indwelling catheters (jugular vein, contralateral to LPS injection site). Serum variables related to metabolism and energy status and inflammation as well as the fecal microbiome, the hepatic metabolome and hepatic mRNA abundance of genes related to mitochondrial FA uptake, activation, translocation, and β-oxidation were reported earlier (Alaedin et al., 2021; Meyer et al., 2021; Tröscher-Mußotter et al., 2021; Xu et al., 2021).

Liver biopsies were taken at d 100 and d 111 PP (d −11 and d 1 relative to LPS administration) under local anesthesia using a biopsy instrument (Bard Magnum, Bard Medica, Karlsruhe, Germany) as described earlier (Alaedin et al., 2021) according to the procedure of Hartwiger et al. (2018). Samples were directly snap-frozen in liquid nitrogen and stored at −80°C until further analysis.

Milk samples were collected at the following times: d 100 PP, 24 h, and 72 h p.i., and d 118 PP. After milking, milk samples were directly kept on ice and stored at −80°C until analysis.

### Blood and Milk Measurements

Milk was first skimmed (4,500 × *g* at 15°C for 30 min) and stored at −20°C thereafter. The Hp and Lf concentrations in serum and skim milk were analyzed by ELISA according to the protocols from Hiss et al. (2004, 2009). The concentrations of Hp in serum were previously published by Meyer et al. (2021). Progesterone was measured in serum using an in-house developed ELISA (Sauerwein et al., 2006). To assess oxidative stress in cows, serum dROM was measured spectrometrically using N,N-diethyl-p-phenylenediamine as chromogenic substrate (Alberti et al., 2000) according to the modified protocol of Regenhard et al. (2014). The resulting values were calculated from the standard curves by linear regression and are expressed as $H_2O_2$ equivalents.

Paraoxonase (PON1) is an antioxidant enzyme associated with high-density lipoproteins (HDL) that has 3 distinct catalytic activities, paraoxonase, lactonase, and arylesterase (Khersonsky and Tawfik, 2006). It can modulate the inflammatory response (James, 2006) and is considered part of the immune system (de Campos et al., 2017). The AEA is regarded as a better surrogate of

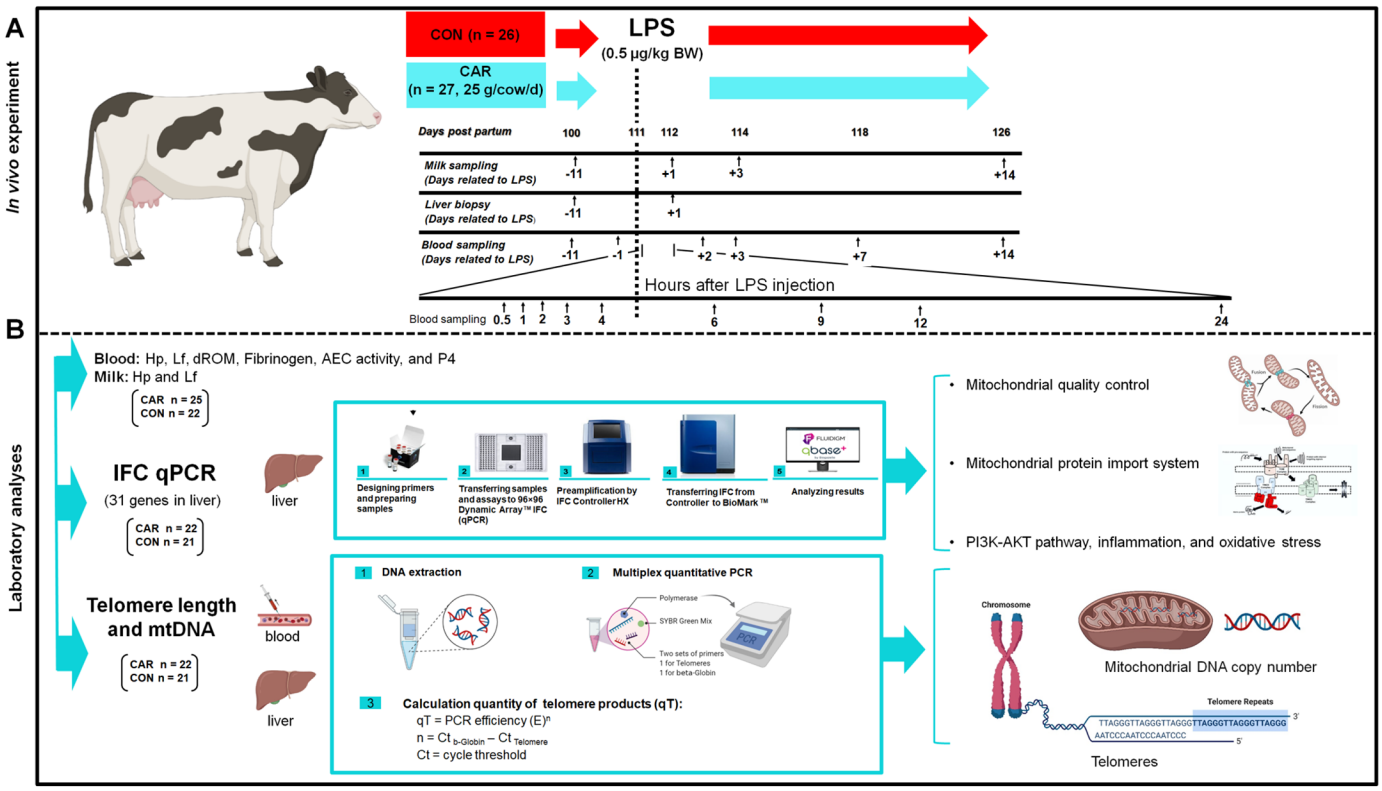


**Figure 1.** (A) Experimental design. Cows in the control group (CON) and cows in the group receiving L-carnitine (CAR) were intravenously injected with 0.5 µg of LPS/kg of BW on d 111 postpartum. The cows in CAR received 125 g of a rumen-protected L-carnitine product (Carneon 20 Rumin-Pro, Kaesler Nutrition GmbH, Cuxhaven, Germany) per cow and day with the feed concentrate; the cows in CON received a rumen-protected fat product (BergaFat F-100 HP, Berg + Schmidt GmbH and Co. KG, Hamburg, Germany) to compensate for the fat content of the mentioned L-carnitine product. The respective supplements were fed from d 42 ante partum until the end of the observation period. Liver biopsies were obtained on d −11 and 1 relative to the LPS administration. Blood samples were collected at the indicated frequency until 14 d after LPS administration. (B) In a total of 43 cows (CAR, n = 22; CON, n = 21), the mRNA abundance of genes involved in mitochondrial quality control (i.e., mitochondrial biogenesis, mitochondrial dynamics, and mitochondrial mitophagy) as well as in the inflammatory response was quantified using microfluidics integrated fluidic circuit chips (96 × 96 dynamic arrays). Liver biopsies from d −1 and 1 relative to the systemic LPS challenge were used to measure telomere length and mitochondrial DNA (mtDNA) copy number in blood and liver. In addition, markers of inflammation and oxidative status (lactoferrin [Lf]: serum and milk; fibrinogen: plasma; haptoglobin [Hp]: milk; derivatives of reactive oxygen metabolites [dROM]: serum, and arylesterase [AEC]: serum) as well as progesterone (P4: serum) were measured. PI3K-AKT = PI3K (phosphatidylinositol 3-kinase) and AKT (protein kinase B); IFC = integrated fluidic circuits.

PON1 concentration than paraoxonase activity (Huen et al., 2009) and was assessed according to Naderi et al. (2011) with minor modifications. In brief, the rate of phenol produced from phenylacetate as substrate was continuously measured at 270 nm, and ARE activity was determined using the molar extinction coefficient of phenol (1310/M per cm) and expressed as KU/L serum (Naderi et al., 2011).

The fibrinogen concentrations in plasma were measured in a turbidimetric assay, imitating the conversion of physiological fibrinogen to fibrin in plasma (Stief, 2008; patent 2008/16) using bovine fibrinogen (#341573, Sigma) as standard. Sample and standard dilutions were pipetted each in duplicate into the wells of a microtiter plate and 100 µL FIFTA reagent containing 60 mg of bovine serum albumin and 0.3 U of bovine thrombin (#605157, Sigma) per mL, were added to each well. Finally, the extinction was measured photometrically at 405 nm at 37°C for 10 min.

The intra- and interassay variances of blood and milk assays measured herein are summarized in Supplemental Table S1 (https://doi.org/10.6084/m9.figshare.14822418.v3; Ghaffari, 2023).

### Telomere Length and mtDNA Copies Per Cell Measurements

The telomere lengths and mtDNAcn were analyzed in blood leukocytes (at d −1 and +2 relative to LPS administration) and liver biopsies. For blood measurements, whole blood was collected with heparinized vacutainers (Vacuette LH Lithium Heparin Tubes,

Greiner Bio-One, Kremsmünster, Austria) and stored at 4°C–8°C immediately after collection until further processing. Genomic DNA was isolated from whole blood and liver tissue biopsies using the Wizard Genomic DNA Purification Kit (Promega, Mannheim, Germany) according to the manufacturer's protocol as described previously (Laubenthal et al., 2016a; Seibt et al., 2022). The DNA yield and purity were assessed for each sample using a NanoDrop 1000 spectrophotometer (peQLab Biotechnology, Erlangen, Germany); DNA integrity was checked in 0.8% agarose gels.

Relative quantities of telomere products (**qT**), being strongly correlated with the actual telomere length, as well as the relative quantity of mtDNAcn from liver cells were measured in the isolated genomic DNA by multiplex quantitative (**q**)PCR according to Cawthon (2009) and as described in detail earlier (Brown et al., 2012; Seibt et al., 2022). Total DNA (10 ng/mL) was mixed with 2 sets of primers each. To determine qT and mtDNAcn, either a primer pair being specific for bovine telomeres (forward 5′–3′: ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT; reverse 5′–3′: TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA; Cawthon, 2009) or 12S rRNA, a gene-specific in the mitochondrial genome (forward 5′–3′: CGCGGTCATACGATTAACCC, reverse 5′–3′: AACCCTATTTGGTATGGTGCTT; Laubenthal et al., 2016b) together with the reference gene β-globin, the housekeeping gene of the chromosomal DNA (forward 5′–3′: CGGCGGCGGGCGGCGCGGGCTGGGCGGGAAGGCCCATGGCAAGAAGG, reverse 5′–3′: GCCGGCCCGCCGCGCCCGTCCCGCCGCTCACTCAGCGCAGCAAAGG; Brown et al., 2012) were used. Before multiplex qPCR measurements, DNA samples were each diluted to 2.5 ng/μL and 10 ng/well were pipetted in triplicate onto the PCR plates (Biostep Thermo Fast 96 PCR detection plates, Thermo Fisher Scientific, Dreieich, Germany). The master mix prepared for multiplex qPCR to measure qT and mtDNAcn per cell consisted of 10 μL primaQuant qPCR SYBR-green low ROX mix (Steinbrenner Laborsysteme GmbH, Wiesenbach, Germany), 2 μL of the mixture of mt 12S rRNA primers (4 pmol/μL each) or 2 μL of the mixture of telomere primers (3.6 pmol/μL), 2.0 μL of the mixture of β-globin primers (2.0 pmol/μL each), and 2 μL of nuclease-free water. Sixteen μL of the master mix were added to 4 μL of sample to give a final volume of 20 μL per well. Nuclease-free water was used as a negative control. A standard curve consisting of a 6-step dilution series (1:5, starting at 60 ng/mL) of pooled blood DNA samples was pipetted in triplicates onto each plate to calculate the efficiency of each multiplex PCR run. Multiplex qPCR was performed on a PCR cycler (Mx3000P Agilent Technologies GmbH and Co. KG, Waldbronn, Germany).

### Calculation of the Relative Telomere Length and mtDNAcn

The qT and mtDNAcn per cell were calculated based on the quantification cycle (Cq) values and the efficiency measurement of each plate for each gene, respectively, as described by Seibt et al. (2022). The relative qT relative to β-globin was calculated as follows: qT = PCR efficiency (E)n, with n = Cq β-globin − Cq telomere (Brown et al., 2012; Laubenthal et al., 2016b). For mtDNAcn per cell, values were calculated as follows: mtDNAcn = β-globin copy number (2 copies per cell) × PCR efficiency (E)-n, with n = Cq mt 12S rRNA − Cq β-globin according to Nicklas et al. (2004) and Laubenthal et al. (2016a).

### RNA Extraction and Reverse-Transcription Quantitative Real-Time PCR

For RNA extraction and cDNA synthesis, samples were handled as previously described by Webb et al. (2019). For all samples, RNA quality was checked by ethidium bromide-denaturing RNA electrophoresis and rechecked for selected samples using a 2100 Bioanalyzer and RNA 6000 Nano Kit system (Agilent) according to the manufacturer's protocol to determine RNA integrity number (7.73 ± 0.34, mean ± SD).

Bovine-specific primer pairs were designed using the National Centre for Biotechnology Information primer blast. A total of 31 target genes from mitochondria, inflammation, and stress response and 8 reference genes were selected. The properties of the primers are listed in Table 1. The primers were designed to have a melting temperature of approximately 60°C. All primers were tested for amplication efficiency in an Mx3000P cycler (Agilent Technologies) with serial dilutions of cDNA obtained from a pool of liver tissue samples according to the minimum information for publication of quantitative real-time PCR experiments guidelines (Bustin et al., 2009). Only primer sets with PCR efficiencies between 90% and 110% and an $R^2$ >0.985 were used for subsequent RT-qPCR experiments. The RT-qPCR analysis was performed using the BioMark HD 96 × 96 system (Fluidigm) as previously described (Alaedin et al., 2021; Ghaffari et al., 2021; Supplemental Figures S1 and S2, and Supplemental Table S1).

Reference genes were determined as described in detail by Alaedin et al. (2021). The optimal number of reference genes was calculated using GeNormplus as a part of qBASEplus. In the present study, 3 reference genes (*LRP10*, *EIF3K*, and *HPCAL*) were determined

**Table 1.** Characteristics of primers used to quantify the target and reference genes[1]

| Gene | Full gene name | Sequence[2] (5′–3′) | NCBI[3] accession no. | Length (bp) |
|---|---|---|---|---|
| Stress response | | | | |
| *TIMP2* | Tissue inhibitor of metallopeptidase 2 | F TCTGGCAACGACATCTACGG | NM_174472.4 | 151 |
| | | R TTCCTCCAATGTCCAGCGAG | | |
| *NFKB1* | Nuclear factor kappa B subunit 1 | F CCCCACGTATGGCGGAATTA | NM_001076409.1 | 110 |
| | | R TGCCACAACTTTCAGGGTCA | | |
| *CP* | Ceruloplasmin | F TTACTCCACTGCCATGTGACC | NM_001256556.1 | 87 |
| | | R TCCAGACTTGATCTCTTCGTTTGG | | |
| *DDIT3* | DNA damage induced transcript 3 | F CCTGAGGAGAGAGTGTTCCAGA | NM_001078163.1 | 70 |
| | | R GATCAGGCTCTGCTTTCAGGT | | |
| *STAT3* | Signal transducer and activator of transcription 3 | F GAGATCAGGGGATGTGGAAGG | NM_001012671.2 | 122 |
| | | R AGGCACTTGACTCACAACGA | | |
| *SOD1* | Superoxide dismutase 1 | F AAAACGGTGTTGCCATCGTG | NM_174615.2 | 121 |
| | | R TTTCCACCTCTGCCCAAGTC | | |
| *CLPX* | Caseinolytic mitochondrial matrix peptidase chaperone subunit X | F TGGAAAAAGTACCCTGAAGAGCC | XM_024997904.1 | 91 |
| | | R AACGGGAAGTCAGTGTCGTG | | |
| *SGK1* | Serum/glucocorticoid regulated kinase 1 | F TCTGGCGATGACGGTGAAAA | NM_001102033.1 | 114 |
| | | R TTCAGGCCCATCCTTCTCTG | | |
| *PDCD4* | Programmed cell death 4 | F GCGGCCTGAGGGAAATACA | NM_001083647.1 | 180 |
| | | R TCCTCAGTCCCAGCATTTTCTT | | |
| *SIRT3* | Sirtuin 3 | F TGGCGTTGTTTCCTCGTTCA | NM_001206669.1 | 132 |
| | | R CACTTGAGGCACCAGCAAAA | | |
| *FGF21* | Fibroblast growth factor 21 | F GATGTTTCGGGGCGTGAATG | XM_005219486.4 | 208 |
| | | R TGGCTAACTGAGGCAGATCG | | |
| *CEPT1* | Choline/ethanolamine phosphotransferase 1 | F TGCACACTGGCAAACATACG | NM_001193130.1 | 107 |
| | | R CTCCAATCACTGCCAGCAAAT | | |
| *EIF4B* | Eukaryotic translation initiation factor 4B | F CCACATCTGGCAGAAATGCAA | NM_001035028.2 | 120 |
| | | R GAGGCTGTTCAGGTTTGGGA | | |
| *UCP2* | Uncoupling protein 2 | F CCTCTACGACTCCGTCAAGC | NM_001033611.2 | 232 |
| | | R GAGTCCCCTAAACCCCTCCT | | |
| Mitochondrial quality control | | | | |
| *TFAM* | Transcription factor A, mitochondrial | F ATGCTTACAGGGCAGACTGG | NM_001034016.2 | 235 |
| | | R TGTGATGTGCCATCCCTAGC | | |
| *FIS1* | Fission mitochondrial 1 | F TAGCGTCTGAGTTGTGAGACC | NM_001034784.2 | 193 |
| | | R ACGGATGTCGTCGTTGTACT | | |
| *MFN1* | Mitofusin 1 | F GAAAGCACAAAGCACAGGGG | NM_001206508.1 | 128 |
| | | R TTTCACTGCTGACTGCGAGA | | |
| *MFN2* | Mitofusin 2 | F CCAGTTGTACCAGCAGACGA | NM_001190269.1 | 97 |
| | | R TCGAGAGAAGAGCAGGGACA | | |
| *OPA1* | Mitochondrial dynamin like GTPase | F CCTTGCAAAATTGGCACCTGA | NM_001192961.1 | 84 |
| | | R CCAGGTGAACCTGTGGTGAA | | |
| *PRKCA* | Protein kinase C alpha | F GACCCCAAGAACGAGAGCAA | NM_174435.1 | 127 |
| | | R AGATTTCCTCGGACAGTCGC | | |
| *PRKN* | Parkin RBR E3 ubiquitin protein ligase | F CCCAGTGGCCATGATAGTGT | XM_024996692.1 | 76 |
| | | R GCTGGTGTCAGAATCGACCT | | |
| Mitochondrial protein import system | | | | |
| *TIM17B* | Translocase of inner mitochondrial membrane 17B | F ATGGAGGAGTATGCTCGGGA | NM_001046488.2 | 85 |
| | | R CACCGCCGATAACACCCATA | | |
| *TIM23* | Translocase of inner mitochondrial membrane 23 | F CCCTTTGGCGGCTGAGTAAT | NM_001083658.1 | 82 |
| | | R AGGTGGTTTTCCAGAAGTGGG | | |

*Continued*

**Table 1 (Continued).** Characteristics of primers used to quantify the target and reference genes[1]

| Gene | Full gene name | Sequence[2] (5′–3′) | | NCBI[3] accession no. | Length (bp) |
|---|---|---|---|---|---|
| *TIM22* | Translocase of inner mitochondrial membrane 22 | F | ACGTGGGCTTTGACCCTAAG | XM_024980096.1 | 229 |
| | | R | AGAACCAGCCCTCTGAAACC | | |
| *TOM20* | Translocase of outer mitochondrial membrane 20 | F | TGCTTGCCTGACGTAGTGTT | NM_001099183.1 | 131 |
| | | R | GCATATCGTCCCAGTCACCG | | |
| *TOM70* | Translocase of outer mitochondrial membrane 70 | F | ATCGCCAGGCATATACAGGA | NM_001075328.1 | 143 |
| | | R | GCCAAACTGCTGTTGATCTGT | | |
| PI3K-AKT signaling pathway | | | | | |
| *AKT1* | AKT serine/threonine kinase 1 | F | ATCCTGGTGAAGGAGAAGGC | NM_173986.2 | 231 |
| | | R | CCGGGACAGGTGGAAGAAAA | | |
| *PDK1* | Pyruvate dehydrogenase kinase 1 | F | AACTCTCCCGAACTAGAACTTGAA | NM_001205957.1 | 228 |
| | | R | ACCTCCTCGGTCACTCATCT | | |
| *PI3K* | Phosphatidylinositol-4,5-bisphosphate3-kinase | F | AGCGCTGAGCAGTGTATCTT | NM_001206047.1 | 197 |
| | | R | GTCCACTGCCCAGATGTCAA | | |
| *PIK3CB* | Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit β | F | AGCGCTGAGCAGTGTATCTT | NM_001206047.1 | 130 |
| | | R | AACCACGGGGCTCTTGAAAT | | |
| *TSC1* | TSC complex subunit 1 | F | CAGGCTGATACAGCAGGGAG | XM_010810529.3 | 95 |
| | | R | CATCTCCGAAGTGGGTCCAG | | |
| Reference gene | | | | | |
| *EIF3K* | Eukaryotic translation initiation factor 3 subunit K | F | CCAGGCCCACCAAGAAGAA | NM_001034489 | 125 |
| | | R | TTATACCTTCCAGGAGGTCCATGT | | |
| *EMD* | Emerin | F | GACTTAGATTCGGCGTCCGT | NM_203361.1 | 140 |
| | | R | TCAGGTTCCCCGTAAGTCCT | | |
| *GAPDH* | Glyceraldehyde-3-phosphate dehydrogenase | F | CCGCATCCCTGAGACAAGAT | NM_001034034.2 | 218 |
| | | R | ATGACGAGCTTCCCGTTCTC | | |
| *HPCAL1* | Hippocalcin like 1 | F | CCATCGACTTCAGGGAGTTC | NM001098964 | 99 |
| | | R | CGTCGAGGTCATACATGCTG | | |
| *LRP10* | LDL receptor related protein 10 | F | TTTTCCCGAATCCTGCCTGT | NM_001100371.1 | 73 |
| | | R | ACAGGCCTCTGTAAGGTGC | | |
| *MARVELD1* | MARVEL domain 1 containing | F | TCGGTGCTTTGATGTCTTGC | NM_001101262.1 | 71 |
| | | R | CAATCCACGGGCACTTCCTA | | |
| *POLR2A* | RNA polymerase II subunit A | F | CTATCGCAGAACCCACTCACC | NM_001206313.2 | 91 |
| | | R | CACAGCGGGAAGGATGTCTG | | |
| *YWHAZ* | Tyrosine 3-mono-oxygenase/tryptophan 5-monooxygenase activation protein zeta | F | CCTACTCCGGACACAGAACAT | NM_174814.2 | 198 |
| | | R | TGACCTACGGGCTCCTACAA | | |

[1]Thermal protocol: initial denaturation for 10 min at 90°C, followed by 40 cycles of amplification (95°C for 30 s, 59°C for 60 s, and 72°C for 60 s).

[2]F = forward; R = reverse.

[3]NCBI = National Center for Biotechnology Information.

as the optimal number of reference genes. The pre-processed Cq values of the target genes were normalized to the values of the reference genes and were used for the statistical analysis of the mRNA data.

### Statistical Analyses

Before analyses, all data were tested for normal distribution using the Kolmogorov-Smirnov test and for homogeneity of variance using Levene's test. Data were subjected to ANOVA using the MIXED procedure of SAS (version 9.4; SAS Institute Inc.), with time as the repeated measure for hepatic mRNA data, relative qT, and mtDNAcn per cell, as well as blood concentrations of Lf, dROM, fibrinogen, P4, AEA, and milk Hp and Lf. All mRNA data were log10-transformed, and for blood and milk data, variables that were not normally distributed were log10-transformed to meet the assumptions of normality and homoscedasticity of the residuals. The model consisted of treatment (CON and CAR), time (representing the LPS effect), and treatment × time interaction as fixed effects and cow as a random effect. Values at d 42 AP were used as covariates in the analysis of blood variables and mRNA data. Three variance-covariance structures (type 1 heterogeneous autoregressive structure, type 1 autoregressive structure, and compound symmetry) were tested, and the type 1 heterogeneous autoregressive covariance structure was determined to be the best-fitting covariance structure for all repeated statements according to the Akaike and Bayesian information criteria. Outliers were identified in the boxplot and by Z-standardization: qT values with SD >2.5 and mtDNAcn per cell values with SD >2.0 were excluded. Statistical significance was set at $P \leq 0.05$, and trends were defined at $0.05 < P \leq 0.10$. Pearson correlation between variables was performed using JASP (JASP Team, 2019).

## RESULTS

### Concentrations of Progesterone and Indicators of Inflammation and Oxidative Status in Serum, Plasma, or Milk

The circulating concentrations of P4, dROM, fibrinogen, and AEA as well as plasma fibrinogen, changed with time but were not affected by the treatment groups (Figure 2A-E). Nine cows (5 CON and 4 CAR) that had P4 values <2.2 ng/mL, indicative of estrus or an inactive corpus luteum on the day before the LPS challenge and 0.5 h p.i., were excluded from the graph (Figure 2E) and the statistical evaluation. In all the remaining cows, the P4 concentrations 30 min p.i. increased as compared with the preceding day, and then decreased to a nadir 6 h p.i. From 7 h onward until d 8 and 16 p.i., baseline values were re-established. The dROM concentrations after LPS administration followed a bimodal pattern with a first peak 2 h p.i., a nadir at 6 and 9 h, and a second increase in the following samples until the end of the observation period (d 126 PP). Fibrinogen increased from the day before LPS to 0.5 h thereafter and then slightly increased further until 72 h p.i. The AEA steadily decreased until 3 to 6 h p.i.; thereafter the values increased again to reach almost basal levels between 9 and 24 h p.i., thereafter a further decline was observed until the last time point considered (i.e., d 126 PP).

The acute-phase protein (APP) Lf increased in both groups after LPS administration, with peak values 2 and 3 h p.i. In this case, group differences were noted (i.e., the CAR group had greater concentrations during the peak phase than the CON group; Figure 2E). An analogous difference was also observed for the Lf concentrations in milk (Figure 3A): Lf increased to a greater extent in the CAR cows. In milk, the greatest values were found later than in blood (i.e., at 72 h p.i.). The concentrations of Hp in serum were reported earlier (Meyer et al., 2021), showing an increase until 48 and 72 h p.i. without any group differences; the Hp values in milk (Figure 3B) increased slightly p.i. and also exhibited no group differences.

### Telomere Length and mtDNAcn

The relative qT and the number of mtDNAcn per cell in blood and liver cells are shown in Table 2. In blood, a time effect was observed for both, the relative qT and mtDNAcn per cell ($P < 0.001$), with increasing numbers of relative qT and decreasing mtDNAcn per cell p.i. In addition, relative qT in blood was 1.2-fold greater in CAR than in CON cows p.i. ($P < 0.05$). In the liver, neither group nor time and group × time interaction affected the relative qT and mtDNA copies per cell.

### Hepatic mRNA Abundance of Genes Involved in Stress Response

The hepatic mRNA abundance of genes involved in stress response are shown in Figure 4. A time effect was observed for *TIMP2* ($P < 0.001$) and *CP* ($P = 0.04$), with greater mRNA abundance after LPS administration, whereas the mRNA abundance for *SOD1* decreased ($P = 0.001$) after LPS administration. Contrary to the CON group, cows supplemented with LC had greater mRNA abundance of *CP* ($P = 0.03$)

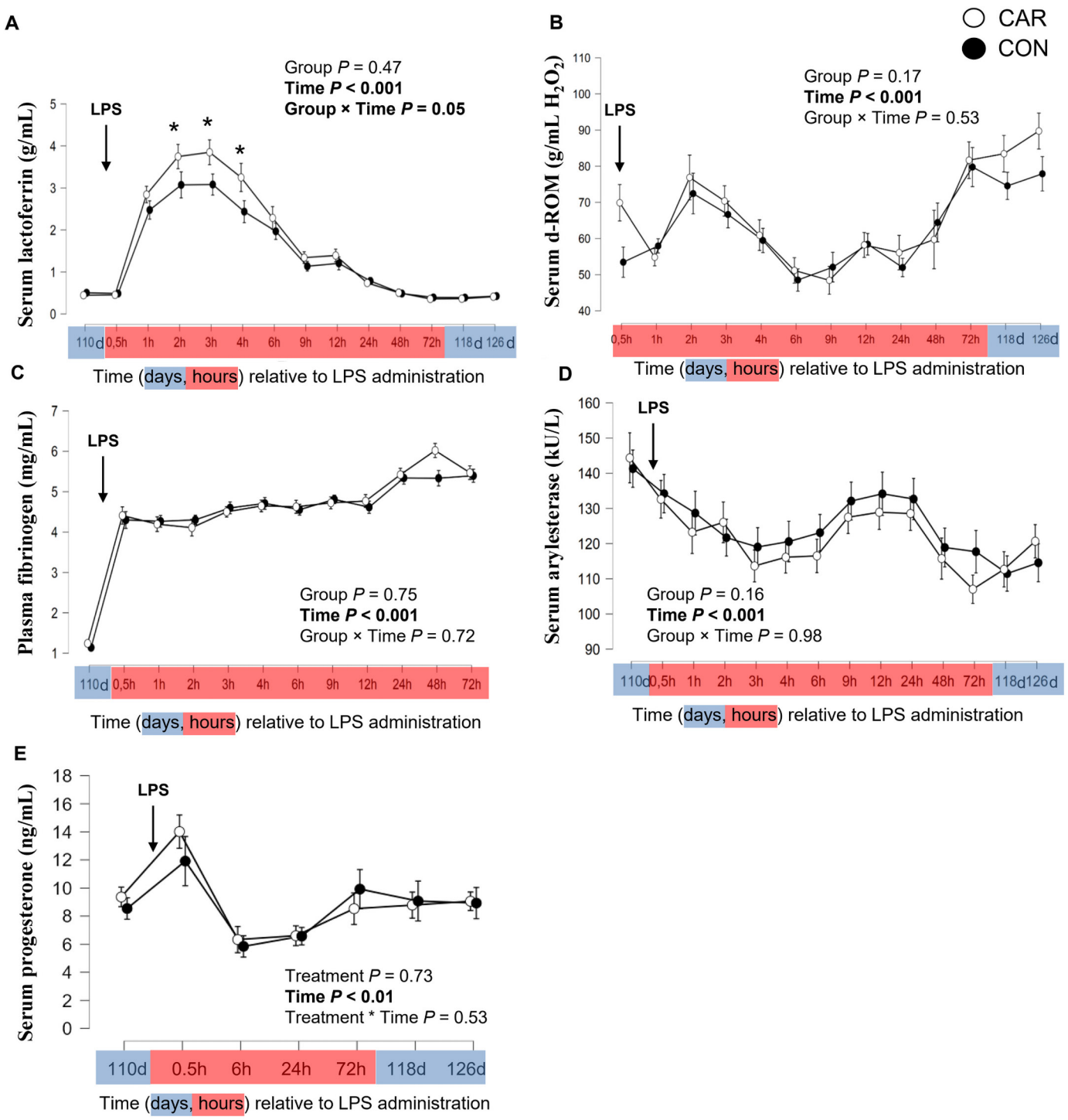


**Figure 2.** Time courses of the circulating concentrations (means ± SEM) of lactoferrin (A), d-ROM (derivatives of reactive oxygen metabolites; B), fibrinogen (C), arylesterase (D), and progesterone (E) in response to a systemic inflammatory challenge (LPS i.v.) administered on d 111 postpartum in cows of the control (CON) and the L-carnitine (CAR) group. (The experimental design is detailed in Figure 1.) Blood samples were frequently taken within the first 3 d (0.5, 1, 2, 3, 4, 6, 9, 12, 24, 48, and 72 h) after LPS administration.

and *SOD1* ($P = 0.01$) after LPS administration and *STAT3* ($P = 0.05$) before and after LPS administration. In addition, the mRNA abundance of *NFKB1* was neither affected by time nor by the group. The mRNA abundance of *CLPX* was greater after LPS administration in both groups ($P = 0.02$).

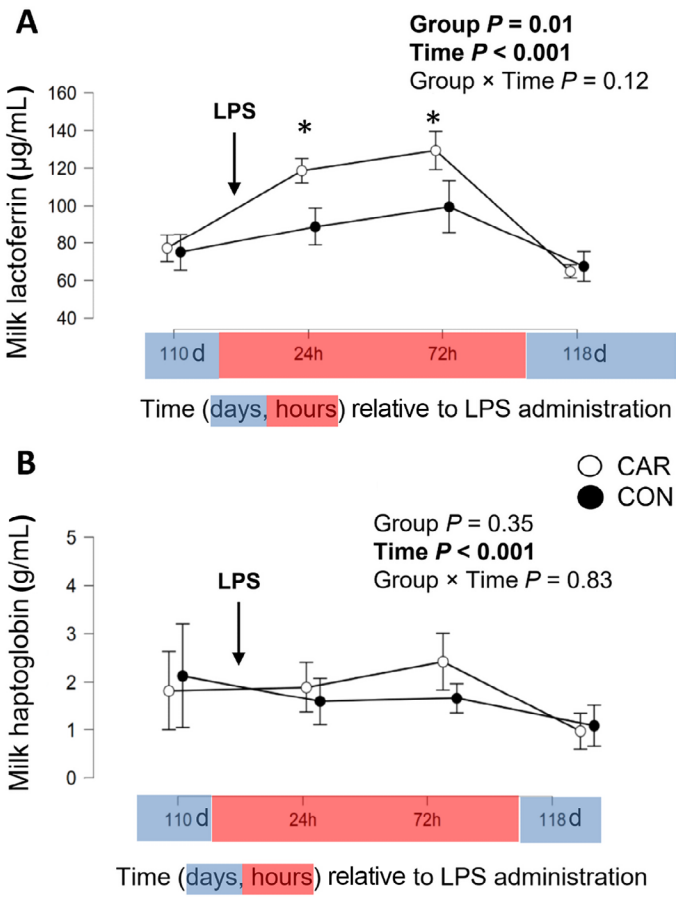


**Figure 3.** Effects of a systemic inflammatory challenge (LPS i.v.) on the concentrations (means ± SEM) of the acute-phase proteins lactoferrin and haptoglobin in milk from cows of the control (CON, n = 21) and the L-carnitine (CAR, n = 22) group (the experimental design is detailed in Figure 1). Milk samples were obtained 1 d before, and 24 and 72 h as well as 8 d after the LPS challenge.

The mRNA abundance of *SGK1*, *FGF21*, *CEPT1*, *SIRT3* (all $P \leq 0.001$), and *UCP2* ($P = 0.02$) changed over time (Figure 4). The mRNA abundance of *SGK1*, *CEPT1*, SIRT3, and *UCP2* increased after LPS administration, whereas that of *FGF21* decreased after LPS administration in both treatment groups. An interaction between group and time was observed for the mRNA abundance of *SOD1*, *CLPX*, *DDIT3*, *SGK1*, *EIF4B*, and *PDCD4*, which had greater mRNA abundances after LPS administration in CAR than in CON cows ($P < 0.05$).

### Hepatic mRNA Abundance of Genes Involved in Mitochondrial Quality Control and the Mitochondrial Protein Import System

None of the mRNA of genes involved in mitochondrial quality control was affected by the group (Figure 5). Regarding mitochondrial biogenesis, an interac-

**Table 2.** Effects of LPS administration on relative quantities of telomeres (qT) and mitochondrial DNA (mtDNA) copy numbers per cell in blood and liver from cows supplemented with (CAR) or without (CON) L-carnitine

| Item | Group | | | | SEM | *P*-value | | |
|---|---|---|---|---|---|---|---|---|
| | CON | | CAR | | | | | |
| | Before LPS (d +100) | After LPS (+48 h) | Before LPS (d +100) | After LPS (+48 h) | | Group | Time | Group × time |
| Blood qT | 469 | 498 | 442 | 525 | 9.08 | 0.82 | 0.001 | 0.05 |
| Blood mtDNA | 88.5 | 70.8 | 91.1 | 79.2 | 1.99 | 0.15 | 0.001 | 0.43 |
| | Before LPS (d +100) | After LPS (+24 h) | Before LPS (d +100) | After LPS (+24 h) | | | | |
| Liver qT | 1,508 | 1,434 | 1,423 | 1,540 | 36.8 | 0.86 | 0.97 | 0.37 |
| Liver mtDNA | 2,508 | 2,085 | 2045 | 1,720 | 118 | 0.10 | 0.12 | 0.99 |

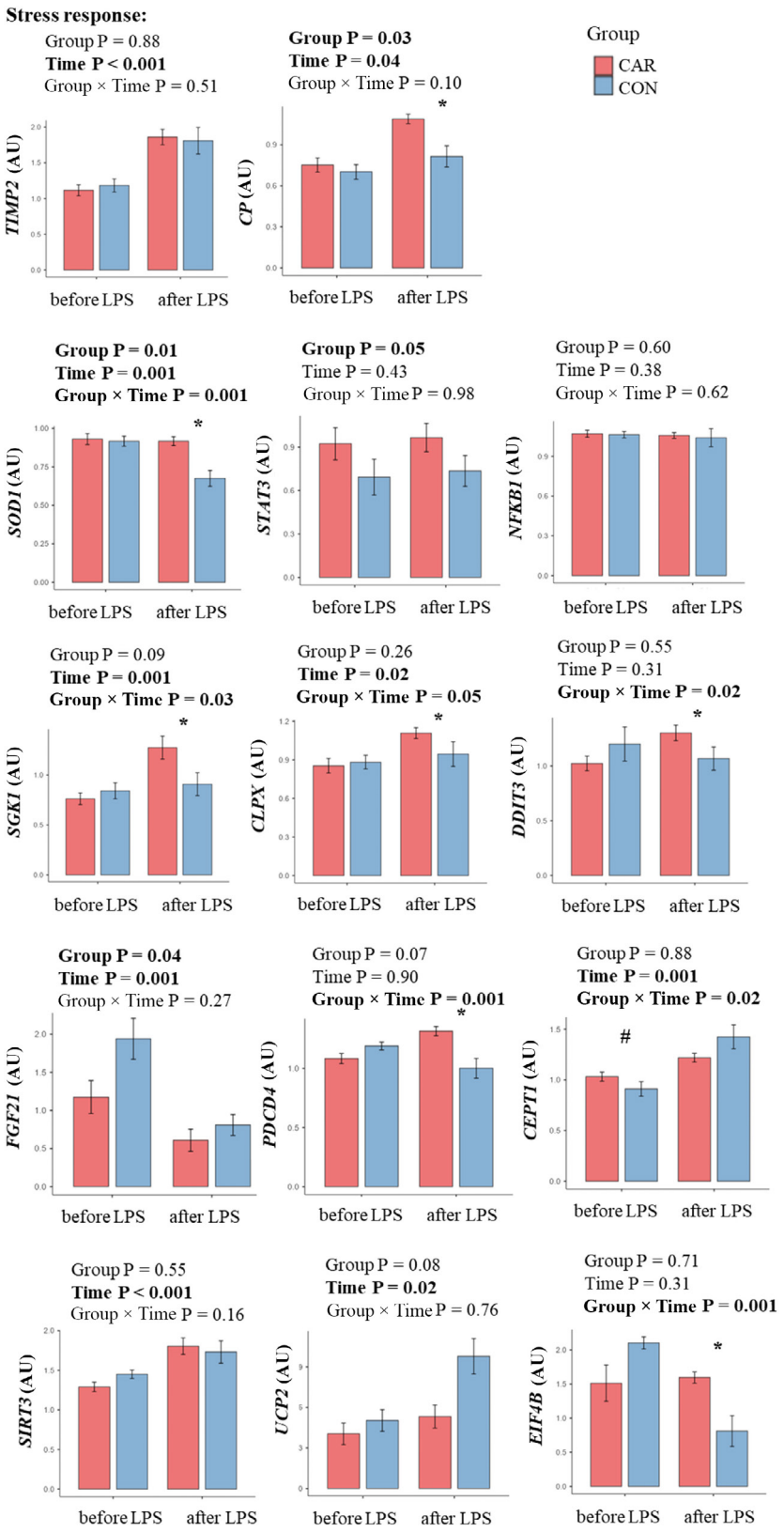


**Figure 4.** Hepatic mRNA abundance (arbitrary units, AU) of genes involved in stress response in control cows (CON, n = 21) and cows with L-carnitine supplementation (CAR, n = 22) before and after the LPS challenge. Data are presented as means ± SEM. * indicates a difference ($P < 0.01$) and # indicates a statistical trend ($0.05 < P < 0.1$) between groups within a time point.

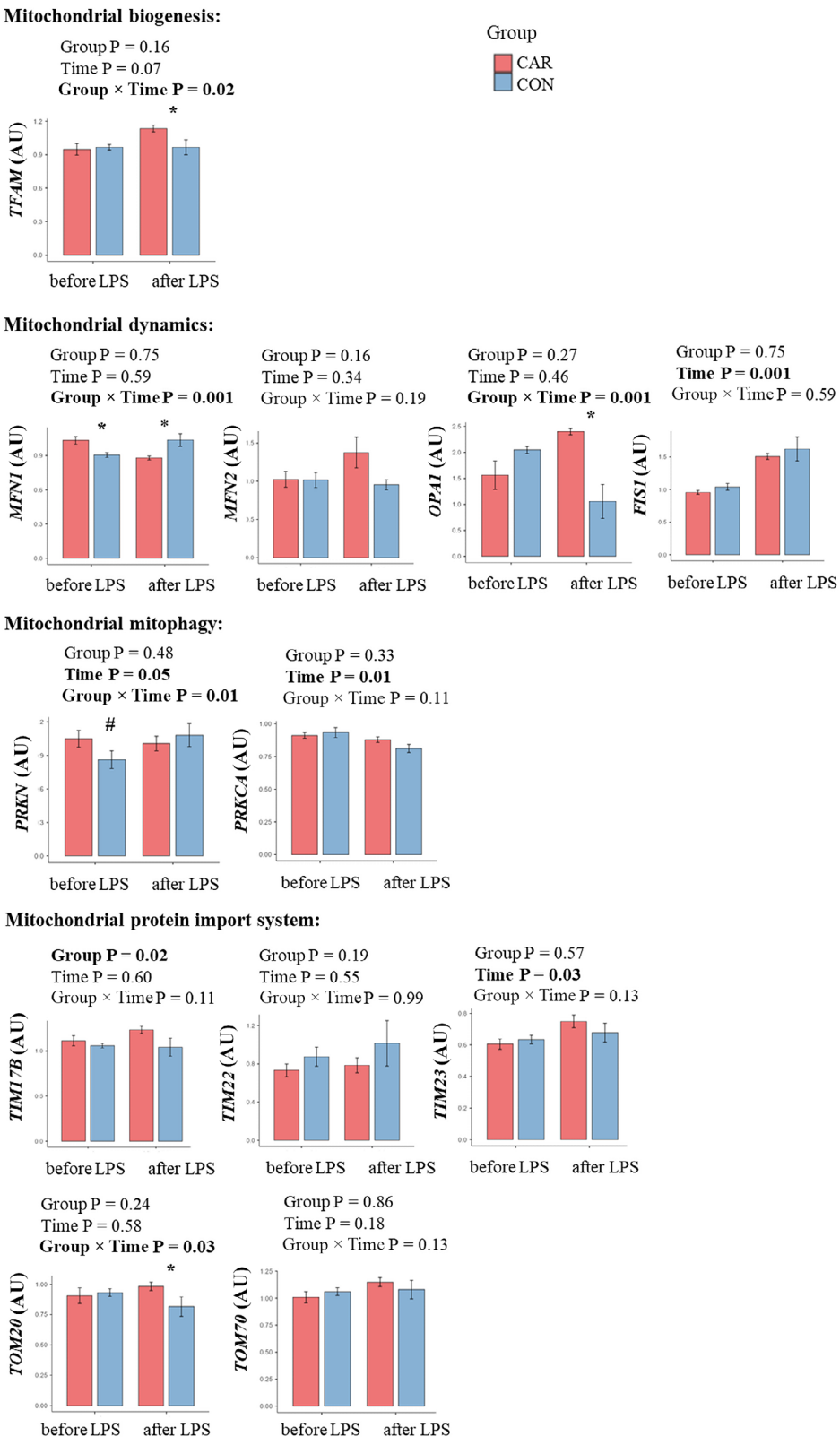


**Figure 5.** Hepatic mRNA abundance (arbitrary units, AU) of genes involved in mitochondrial quality control (i.e., mitochondrial biogenesis, mitochondrial dynamics, and mitochondrial mitophagy) as well as in the mitochondrial protein import pathway in nonsupplemented cows (CON, n = 21) and cows supplemented with L-carnitine (CAR, n = 22) before and after an LPS challenge. Data are presented as means ± SEM. * indicates a difference ($P < 0.01$) and # indicates a statistical trend ($0.05 < P < 0.1$) between groups within a time point.

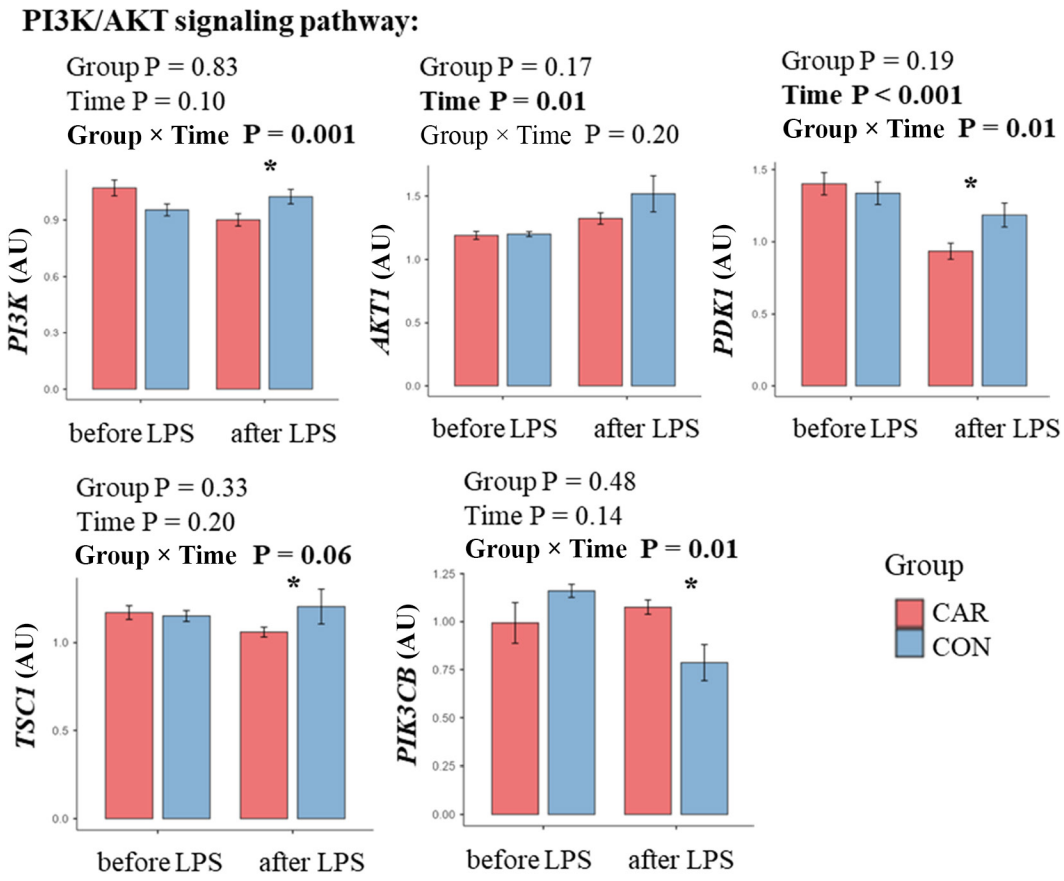


**Figure 6.** Hepatic mRNA abundance (arbitrary units, AU) of genes involved in the PI3K-AKT-signaling pathway in nonsupplemented cows (CON, n = 21) and cows supplemented with L-carnitine (CAR, n = 22) before and after an LPS challenge. Data are presented as means ± SEM. * indicates a difference ($P < 0.01$) between groups within a time point.

tion between group and time was observed for *TFAM* ($P = 0.02$), and the mRNA abundance of *TFAM* was increased after LPS administration, with a tendency for greater abundance in CAR than in CON cows ($P = 0.07$). For *FIS1* and *PRKN*, the mRNA abundance was increased after LPS administration ($P < 0.05$), whereas that of *PRKCA* decreased after the LPS challenge ($P = 0.01$). Interaction between group and time was observed for the mRNA abundance of the regulators of mitochondrial dynamics, *MFN1* ($P = 0.001$; decreased in CAR cows and increased in CON cows) and *OPA1* ($P = 0.001$; increased in CAR cows, decreased in CON cows), as well as for the mRNA abundance of *PRKN* ($P = 0.01$), which is involved in mitochondrial mitophagy. The mRNA abundance of *PRKN* tended to be lesser in CON cows than in CAR cows before LPS administration.

For genes involved in the mitochondrial protein import system (Figure 5), a group effect was observed only for the mRNA abundance of *TIM17B*, which increased in CAR cows ($P = 0.02$). In addition, time only affected the mRNA abundance of *TIM23*, with mRNA abundance increasing after LPS administration ($P = 0.03$). There was an interaction between group and time only for *TOM20*, with lesser mRNA abundance in CON compared with CAR cows after LPS administration ($P = 0.03$).

### Hepatic mRNA Abundance of Genes Involved in the PI3K/AKT Signaling Pathway

The mRNA abundance of genes involved in the PI3K/AKT1 signaling pathway was not affected by the group (Figure 6). A time effect was observed for *AKT1* with increasing mRNA abundance after LPS administration ($P = 0.01$) and for *PDK1* with decreasing mRNA abundance after LPS administration ($P < 0.001$). There was an interaction between group and time for the mRNA abundance of *PDK1* ($P = 0.01$) and *PI3K* ($P = 0.001$; both with greater abundance in CON than in CAR cows), for *PIK3CB* ($P = 0.01$; lesser abundance in CON than in CAR cows)

as well as a trend for the mRNA abundance of *TSC1* ($P = 0.06$; greater abundance in CON than in CAR cows) after LPS administration.

## DISCUSSION

The dietary supplementation with LC was effectively increasing the serum concentrations of carnitine and its metabolites (Meyer et al., 2021). Particularly during the first hours after LPS injection, all cows developed clinical signs and symptoms characteristic of an inflammatory response as described in detail by Meyer et al. (2021). Our hypothesis that dietary supplementation with LC will affect the response to an inflammatory stimulus in terms of telomere length, mtDNAcn, mRNA abundance of genes related to mitochondrial function and cellular stress as well as various indicators of inflammation and oxidative status was confirmed for telomere length in blood leukocytes, the mRNA of 14 out of 31 target genes investigated (as indicated by significant group × time interactions), and for the Lf concentrations in serum and milk.

### *Effects of LC Supplementation on Progesterone and Indicators of Inflammation and Oxidative Stress Assessed in Blood and Milk*

The inflammatory response to LPS in this study was already demonstrated by clinical signs and elevated serum concentrations of Hp. Except for rumen motility, the groups were not differently reacting (Meyer et al., 2021). To further extend the response variables, we herein measured P4, lactoferrin, fibrinogen, dROM, and AEA in blood, and lactoferrin as well as Hp in milk.

The initial increase in P4 in the first 3 h after LPS and a decline thereafter is in line with results observed in nonlactating, nonpregnant cows (Herzog et al., 2012). Treatment with LPS has been demonstrated to reduce the secretion of LH in sheep (Coleman et al., 1993). According to in vitro studies on isolated perfused bovine ovaries, the LPS-related decrease of P4 secretion was mostly attributable to an increase in LPS-induced apoptosis rather than decreased steroidogenesis (Lüttgenau et al., 2016). Moreover, Mohammed et al. (2020) demonstrated that luteal vasculature is compromised in response to LPS. The initial increase in P4 observed herein and by Herzog et al. (2012) in vivo after LPS administration might be related to adrenal P4 synthesis, which has been demonstrated to increase after LPS in ovariectomized heifers (Kujjo et al., 1995).

Lactoferrin is a nonheme iron-binding glycoprotein with the greatest concentrations in milk but it is also found in the blood. In addition to the iron-binding ability, Lf binds to the lipid A of LPS and may thus inhibit the effect of endotoxin (Appelmelk et al., 1994; Elass-Rochard et al., 1995). Through its interaction with specific receptors on monocytes/macrophages and other immune and nonimmune cells, Lf attenuates inflammation and contributes to tissue repair; moreover, Lf was demonstrated to protect against oxidative stress-induced cellular damage (Kruzel et al., 2017). Upon inflammation, the monocyte/macrophage system produces inflammatory mediators, which in turn induce a generation of new immune cells and also the degranulation of mature neutrophils; subsequently, Lf is massively released from the neutrophil's secondary granules (Kruzel et al., 2017). The neutrophil (granulocyte) counts in the present study increased and peaked 4 h after LPS but were not different between the CON and the CAR group (Kononov et al., 2022). The time course of the Lf concentrations in blood followed the same pattern with greater values in the CAR group from 2 to 4 h after LPS. Neutrophil activation and function also rely on FA utilization and oxidation (Curi et al., 2020), and the elevated supply of LC may have facilitated the intracellular metabolism of FA. However, the greater increase in LF in the CAR group is likely not due to a greater extent of degranulation, because (a) inhibition of OXPHOS was demonstrated to leave the release from neutrophils unaffected (Smith et al., 1983), and (b) mitochondrial FA oxidation is largely limited to immature neutrophils to support NADPH-oxidase-dependent ROS production when glucose utilization is restricted to ensure neoplastic cell growth (Rice et al., 2018). We thus speculate that the greater Lf concentrations in the CAR group might be due to greater incorporation of Lf during maturation and consequently a greater content in the granulae, which was released upon LPS. The pattern in milk with elevated concentrations in the CAR group is in line with this notion.

Haptoglobin, a further APP, is mainly of hepatic origin and not released from neutrophils. We found the expected increase of Hp in blood with a peak at 48 to 72 h, but no differences between the groups (Meyer et al., 2021). In milk, time was significant, but no group difference was observed.

In various pathological conditions or diseases, fibrinogen, the end product of the coagulation cascade, modulates inflammatory processes and is considered as a positive acute-phase reactant required in the early phase of systemic inflammation (Davalos and Akassoglou, 2012). Supplementation with LC was shown to reduce plasma fibrinogen in human hemodialysis patients (Hakeshzadeh et al., 2010) and in rats with

metabolic syndrome (Zayed et al., 2021). However, in the current trial, we found no difference in the response to LPS between the CAR and the CON group. In addition, platelet count and platelet-related as well as erythrocyte-related parameters in the cows of this trial were not affected by the CAR supplementation from wk 6 AP to wk 15 PP (Kononov et al., 2022).

L-Carnitine is part of the FA uptake shuttle in mitochondria (Longo et al., 2016) and can counteract inflammation-induced oxidative stress (Schlegel et al., 2013). To characterize oxidative stress, we determined dROM photometrically in serum. In direct response to LPS administration, dROM increased after injection. Considering that mitochondria are important contributors to cellular ROS (Sullivan and Chandel, 2014), the decreasing dROM concentrations 2 h after LPS administration might be related to the decrease in mtDNAcn per blood cell (see discussion on "Effects of LC Supplementation on mtDNAcn, Mitochondrial Quality Control, and Protein Import System") though the lack of correlation between dROM and mtDNAcn is not in support of the latter notion.

The enzyme PON1 is primarily expressed in the liver and released into the circulation, where it binds high-density lipoprotein and protects low-density lipoproteins from oxidation (Hashemi et al., 2011). In cows with fatty liver, serum PON1 activity was reduced as measured by the activity of PON, lactonase, and AEA (Farid et al., 2013). Moreover, in periparturient dairy cows, plasma PON1 activity was reduced in response to inflammatory conditions, indicated by increased plasma Hp concentrations in parallel to a substantial decrease in milk yield (Bionaz et al., 2007). The observation of decreasing serum AEA activity after LPS administration in the present study is in line with the aforementioned studies.

### Relative Telomere Lengths in Response to Systemic Inflammation

In the companion study, total leukocyte count was determined as an indicator of acute-phase response (Meyer et al., 2021). The total leukocyte count reached its minimum 3 h after LPS administration, followed by an increase until 24 h p.i., and a further decline to basal levels at d 7 p.i. that were maintained until the end of the observation period (Meyer et al., 2021). In humans, progressive shortening of telomeres is associated with an increased incidence of disease (Shammas, 2011) and chronic inflammation (Wolkowitz et al., 2011). However, little is known about the effects of inflammation on telomere length in dairy cows. Herein, we examined the effect of an acute inflammation on leukocyte and liver telomere length in mid-lactation cows. The increase in total leukocyte counts until 24 h p.i. (Meyer et al., 2021) implies the formation of new leukocytes, which in turn may explain increasing relative qT after LPS administration. Oxidative stress due to inflammation damages DNA, proteins, and lipids (Sordillo and Aitken, 2009) and therefore should also affect telomere length. Increasing relative qT in the present study could arise from newly formed leukocytes, though we cannot distinguish between persisting and newly generated leukocytes.

Considering the benefical effects of LC on inflammatory markers and oxidative stress (Fathizadeh et al., 2020), telomere length might also be affected because oxidative stress and inflammatory diseases have been proposed to be associated with accelerated telomere shortening and dysfunction (Barnes et al., 2019; Heba et al., 2021). However, in the various indicators of oxidative and inflammatory status assessed herein we found group differences only for the Lf concentrations in both serum and milk. The greater relative qT in leukocytes from CAR cows compared with CON cows p.i. nevertheless indicate that LC may attenuate telomere shortening. This is in line with in vitro studies on cardiac differentiated bone marrow resident CD117+ stem cells demonstrating that LC increased the expression of telomerase reverse transcriptase and also elongated the telomere length (Fathi et al., 2020).

### Effects of LC Supplementation on the mRNA Abundance of Hepatic Genes Related to Stress Response

Mediated by cytokines, an APR is activated as a result of infection or trauma, leading to elevated hepatic gene transcription of acute-phase reactants including CP (Ceciliani et al., 2012). We suggest, that greater mRNA abundance of *CP* after LPS administration in cows supplemented with LC in their diets, was due to a stimulation of hepatocytes by LC as described in broiler chickens when LC supplementation seemed to modulate the innate immune response in terms of increased circulating APP (Buyse et al., 2007).

Regulating a large number of genes involved in different processes of immune and inflammatory responses, the nuclear factor-κB (NF-κB) transcription factor plays an essential role in innate and adaptive immune responses, cell proliferation, apoptosis, and tumorigenesis (Liu et al., 2017). In the present study, signs of an APR in which NF-κB activates cytokines were detected. However, the LPS administration did not affect the hepatic mRNA abundance of *NFKB1*, neither in cows supplemented with LC nor in control cows. As

described by Meier-Soelch et al. (2021), activation of the NF-κB pathway as well as the expression of NF-κB target genes can be highly variable; in addition, activity levels after the NF-κB response are highly dynamic, thus the reliable detection limit may be too low (Meier-Soelch et al., 2021). Considering this, the activation of the APR through LPS may not run exclusively at a transcriptional level.

The signal transducer and activator of transcription 3 (STAT3) is thought to be closely associated with NF-κB, co-regulating several inflammatory genes, and required for immunosuppressive effects (Yu et al., 2009). Therefore, the greater mRNA abundance of *STAT3* in CAR cows could contribute to an improved immune response in the liver. The tissue inhibitor of the metalloproteinase family TIMP-2, is involved in the regulation of multifunctional metalloproteinases and can directly upregulate the transcriptional activity of NF-κB in melanoma cells (Sun and Stetler-Stevenson, 2009). Because apoptosis is regulated by NF-κB signaling, overexpression of *TIMP2* could protect cells from apoptosis (Sun, 2010). Therefore, increasing mRNA abundance of *TIMP2* after LPS administration in this study could protect hepatocytes from LPS-induced inflammation.

DDIT3 is a key regulator of stress responses and is induced by DNA damage, ER stress, hypoxia, and starvation (Jauhiainen et al., 2012). Conversely, suppression of DDIT3 has been reported to induce ER stress and inflammatory responses (Kim et al., 2017). Therefore, the decreased mRNA abundance of *DDIT3* in CON cows after LPS administration may indicate that hepatocytes are more susceptible to the LPS-induced inflammatory response when compared with CAR cows. In addition, the mRNA abundance of *SOD1*, an enzyme important for tissue defense against oxidative stress (Thorpe et al., 2013), was greater in CAR cows compared with CON cows, pointing to the local protection against oxidative stress in CAR cows.

Fibroblast growth factor 21 (FGF21) contributes to the regulation of the metabolic and stress adaptations during nutrient deprivation and promotes hepatic FA oxidation, ketogenesis, and gluconeogenesis (Badman et al., 2007; Gessner et al., 2017). In addition to the physiological roles of FGF21 in maintaining energy homeostasis, FGF21 serves as a stress hormone, increasing the availability of energy substrates to cope with energy deprivation or stress (Kim and Lee, 2014). In contrast to early-lactating dairy cows with an increased hepatic expression of *FGF21* after LPS-induced systemic inflammation (Akbar et al., 2015), the hepatic mRNA abundance of *FGF21* was decreased in both groups after LPS administration in the current study. Cows had an increased stress response with increased cortisol concentrations directly after the LPS administration (Meyer et al., 2021); however, 24 h after LPS administration, cortisol concentrations decreased to the level before LPS administration. Moreover, decreasing mtDNAcn in blood in the present study was accompanied by a temporary decrease of dROM, 2 h after LPS administration. Therefore, decreasing *FGF21* mRNA abundance in mid-lactating cows might be accompanied by temporary attenuated mitochondrial stress directly around 24 h after LPS administration.

An increase in uncoupling protein 2 (*UCP2*) mRNA abundance after LPS administration could regulate both mitochondrial ATP production and ROS generation (Toda and Diano, 2014). As a sensor of mitochondrial oxidative stress, UCP2 is important in controlling mitochondrial ROS generation (Donadelli et al., 2014). In addition, UCP2 has been suggested to regulate the activity of SIRT3 by sensing energy levels to maintain a mitochondrial steady state (Su et al., 2017). Under stressful conditions such as fasting and exercise, SIRT3 controls the energy demand by deacetylating and acetylating mitochondrial enzymes (Ansari et al., 2017). In cows with fatty liver, *SIRT3* mRNA abundance was downregulated in the liver, suggesting the involvement of SIRT3 in the induction of hepatic lipid accumulation (Liu et al., 2020). However, mid-lactating cows in the present study had decreased liver triglyceride contents in response to LPS (Alaedin et al., 2021). Increasing mRNA abundance of *SIRT3* after LPS administration could therefore exhibit beneficial effects on hepatocytes during inflammation.

### Effects of LC Supplementation on mtDNAcn, Mitochondrial Quality Control, and Protein Import System

Inflammatory responses are associated with mitochondrial dysfunction (López-Armada et al., 2013). Increased ROS levels from dysfunctional mitochondria lead to cellular damage (Giorgi et al., 2018) and are thought to be a major cause of aging (Barja, 2014). In addition, elevated ROS were associated with mitochondrial oxidative damage, along with reduced mtDNAcn (Lee et al., 2014). In the present study, increased levels of free FA after LPS administration in both groups (Meyer et al., 2021) could contribute to the decrease in mtDNAcn in blood, because free FA knowingly enhance the synthesis of mitochondrial ROS (Schönfeld and Wotjczak, 2008). However, liver mtDNAcn did not seem to be affected, because liver triglyceride content decreased in response to LPS as shown previously for cows of the current study (Alaedin et al., 2021). The increase in hepatic mtDNAcn from AP values to 100 d PP (data not shown) may be related to the increased

metabolic activity and energy requirements of hepatocytes during lactation, as shown by Laubenthal et al. (2016a) in early-lactating dairy cows.

As a protecting system, a comprehensive set of adaptive quality control mechanisms can optimize the overall number, distribution, and function of mitochondria (Cherry and Piantadosi, 2015). Suliman et al. (2003) reported increased expressions of hepatic genes related to mitochondrial biogenesis as well as stimulation of mtDNA replication after LPS administration in cultured rat hepatoma cells. Increased mRNA abundance of *TFAM*, one of the regulators of mitochondrial biogenesis, after LPS administration in the present study, might balance the (unchanged) number of mtDNAcn in liver, thus counteracting mitochondrial depletion through LPS. This is supported by the increased mRNA abundance of *CLPX*, which can regulate mtDNAcn by increasing the DNA-binding capacity of TFAM (Kasashima et al., 2012).

During metabolic or environmental stress, mitochondria dynamically adapt through fusion and fission to maintain mitochondrial function: stressed mitochondria fuse to separate the healthy and damaged mitochondria through the fission mechanism (Yue and Yao, 2016). Mitochondrial fission is regulated by dynamin 1-like and fission 1 (FIS1), while fusion processes are controlled by optic atrophy 1 (OPA1) and mitofusin 1 and 2 (MFN1 and MFN2; Yue and Yao, 2016). The reduction of FIS1 and OPA1 leads to extensive mitochondrial fragmentation and significantly protects against senescence-related changes (Lee et al., 2004). Of the genes involved in mitochondrial dynamics, *FIS1* mRNA increased in both groups after LPS administration, indicating increased mitochondrial fission due to increased oxidative stress. Damaged mitochondria can be selectively removed by mitophagy as part of the mitochondrial quality control mechanism to regulate the cellular mitochondrial population (Wang and Klionsky, 2011). Parkin (PRKN) has been linked to the regulation of stress-induced mitophagy in vivo (Sliter et al., 2018). We suggest that an increase in *PRKN* mRNA abundance following LPS administration in CON cows may upregulate mitophagy. Protein kinase C delta (PRKCD) has been proposed as a regulator of PRKN-independent mitophagy (Munson et al., 2021). Decreasing *PRKCA* mRNA abundance after LPS administration in both groups, therefore, suggests regulation of mitophagy by PRKN rather than PRKCA.

Because >99% of mitochondrial proteins are encoded by the nuclear genome and synthesized in the cytosol, mitochondrial protein import is fundamental to normal mitochondrial physiology (Needs et al., 2021). The nuclear genome contains most of the proteins required for the assembly and function of respiratory complexes, which means that without fully functional protein import, mitochondrial respiration is disrupted and the main cellular ATP source is depleted (Needs et al., 2021). Mitochondrial proteins are imported into the mitochondrial matrix by passing the outer (TOM complex) and the inner (TIM complex) membranes (Harbauer et al., 2014). The mitochondrial protein import system can be regulated at various levels related to signaling, cellular metabolism, stress, and disease (Harbauer et al., 2014). In the present study, we observed no effect of LPS administration on the mRNA abundance of the TOM (*TOM70* and *TOM20*) complex and *TIM22* genes. However, LPS-induced upregulation of *TIM23* genes was observed in both groups and upregulation of *TIM17B mRNA* by LC, suggesting that the inner mitochondrial membrane translocases are more sensitive than the outer ones. This is consistent with the findings of Ghaffari et al. (2021) who showed that the transition from late gestation to early lactation was associated with changes in mRNA abundance of genes related to the TIM complex (*TIM22* and *TIM23*) in the mitochondrial protein import system in the liver of dairy cows.

### Effects of LC Supplementation on Hepatic Genes Related to the PI3K/AKT Signaling Pathway

Intracellular PI3K/AKT signaling is involved in several processes, including cell proliferation and survival, as well as glucose metabolism (Vivanco and Sawyers, 2002). Triggered by extracellular signals under physiological conditions, PI3K is activated and subsequently stimulates the serine/threonine kinase AKT at the cell membrane (Vivanco and Sawyers, 2002). Overactivation of the PI3K/AKT pathway results in significant dysregulation of normal cellular functions and has been linked to various diseases in humans (Keppler-Noreuil et al., 2016). However, selective inhibition of PI3K reduced the severity of inflammation in mouse disease models (Hawkins and Stephens, 2015). In the present study, dietary supplementation of LC reduced the mRNA abundance of *PI3K* following LPS administration, which could diminish the inflammatory response to LPS. Increased mRNA abundance of *TSC1* and *PDK1*, serving as substrates for AKT, after LPS administration in CON cows, may be related to mechanisms preventing exaggerated immune responses postulated in the context of the PI3K/AKT/mTORC1 kinase pathway (reviewed by Mezzetti et al., 2020).

## CONCLUSIONS

After LPS administration, mtDNAcn per cell was neither affected in blood leukocytes nor liver. Telomere

length was increased in response to LPS-induced systemic inflammation only in blood leukocytes but not in liver tissue. This increase was likely attributable to the recruitment of new neutrophils into the blood-stream in response to LPS. The increase in serum and milk Lf concentrations may represent an adaptive mechanism to attenuate the LPS-induced inflammation. The mRNA data pointed toward a modulatory effect of LC on mitochondrial dynamics with an increase in mitochondrial fission due to increased inflammation-induced oxidative stress. The LPS administration altered the mRNA abundance of key genes related to mitochondrial protein import, whereby LC supplementation modified in particular the inner mitochondrial membrane translocases (TIM complex). Taken together, these results may point to a protective effect of LC against LPS-induced inflammation.

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